The Survival of *Escherichia coli* O157:H7 in the Presence of *Penicillium expansum* and *Glomerella cingulata* in Wounds on Apple Surfaces†

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ABSTRACT

The survival of *Escherichia coli* O157:H7 in the presence of one of two plant pathogens, *Penicillium expansum* and *Glomerella cingulata,* in wounds on apples was observed during 14 days storage at room temperature (RT) and at 4°C. The aim of this work was to determine if changes in apple physiology caused by the proliferation of fungal decay organisms would foster the survival of *E. coli* O157:H7. Trials were performed where (A) plant pathogens (4 $\log_{10}$ spores) were added to apple wounds 4 days before the wounds were inoculated with *E. coli* O157:H7 (3 $\log_{10}$ CFU g^{-1} apple) (both RT and 4°C storage), (B) plant pathogens and *E. coli* O157:H7 were added on the same day (both RT and 4°C storage), and (C) *E. coli* O157:H7 was added 2 days (RT storage) and 4 days (4°C storage) before plant pathogens. In all trials *E. coli* O157:H7 levels generally declined to <1 $\log_{10}$ at 4°C storage, and in the presence of *P. expansum* at 4°C or RT. However, in the presence of *G. cingulata* at RT *E. coli* O157:H7 numbers increased from 3.18 to 4.03 $\log_{10}$ CFU g^{-1} in the apple wound during trial A, from 3.26 to 6.31 $\log_{10}$ CFU g^{-1} during trial B, and from 3.22 to 6.81 $\log_{10}$ CFU g^{-1} during trial C. This effect is probably a consequence of the attendant rise in pH from 4.1 to approximately 6.8, observed with the proliferation of *G. cingulata* rot. Control apples (inoculated with *E. coli* O157:H7 only) were contaminated with opportunistic decay organisms at RT during trials A and B, leading to *E. coli* O157:H7 death. However, *E. coli* O157:H7 in control apples in trial C, where no contamination occurred, increased from 3.22 to 5.97 $\log_{10}$ CFU g^{-1}. The fact that *E. coli* O157:H7 can proliferate in areas of decay and/or injury on fruit highlights the hazards associated with the use of such fruit in the production of unpasteurized juice.

The noted high acid tolerance of *Escherichia coli* O157:H7 *(4, 8, 12, 16, 22, 23)* has resulted in the implication of this organism in foodborne disease outbreaks involving unpasteurized apple cider *(2, 6, 7),* a product not previously considered a food safety risk due to its high acidity. These outbreaks have led the Food and Drug Administration to mandate that all juice processors either place a warning statement on packaged juices not processed in a manner guaranteed to yield at least a 5-$\log_{10}$ reduction in target pathogenic organisms, such as *E. coli* O157:H7 and *Salmonella,* or have in place a hazard analysis critical control point system that can guarantee a 5-$\log_{10}$ reduction of target pathogenic organisms in the finished product *(11).* This mandate has led to a considerable amount of research into the safety of fruit juices, including apple cider.

The exact mode of contamination of apple cider has been difficult to determine in outbreaks to date. Potential sources include irrigation water, manure, sewage, poor worker hygiene, harvesting equipment and containers, insects, birds, and processing equipment *(3).* Much attention has been centered on the use of drops, i.e., apples that had fallen on the ground prior to harvest, that may have been used in production of the implicated ciders. Such apples are a potential vector for *E. coli* O157:H7, as they may be contaminated with feces from animals such as deer *(14)* and birds *(21)* that have been known to harbor *E. coli* O157:H7. Drops may also have pockets of fungal decay, in which the growth and metabolism of opportunistic plant pathogens will lead to changes in the physiology of the apple. This may, in turn, improve the growth potential of *E. coli* O157:H7, e.g., by increasing the pH of the apple in that area. The minimum growth pH for *E. coli* O157:H7 is 4.0 to 4.5 *(8);* therefore, the pH of an apple, generally <pH 4.0, is at the lower limit of growth for this organism.

It is important to recognize any situation in which an increase in apple pH, which has the potential to create a more favorable environment for *E. coli* O157:H7, may occur. The purpose of this study was to investigate the survival of *E. coli* O157:H7 in the presence of two apple diseases, blue mold rot and bitter rot, caused by *Penicillium expansum* and *Glomerella cingulata,* respectively.

MATERIALS AND METHODS

***E. coli* O157:H7.** *E. coli* O157:H7 strain Sea 13B88 (from an outbreak associated with apple cider in the northwest United States in 1996) *(6)* was obtained from the Microbial Food Safety research unit culture collection (Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, Pa.) and stored in 20% glycerol at −80°C. To resuscitate, the organism was grown in 10 ml tryptic soy broth (TSB; Difco, Detroit, Mich.) at 37°C

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for 8 h, at which point a 100-μl aliquot was transferred to 100 ml TSB with glucose (TSB supplemented with glucose to a final concentration of 1% wt/vol). Cultures were grown at 37°C for 18 h. Supplementation of TSB with glucose in this manner has been reported to be an effective method of inducing pH-dependent stationary-phase acid resistance in *E. coli* O157:H7 *(5)*. Cells were recovered by centrifugation (11,170 × g at 4°C for 10 min) (Sorvall Instruments, Newtown, Conn.) and washed once in sterile 0.1% (wt/vol) peptone water (PW; Difco). The cell pellet was resuspended in 100 ml sterile PW, to a final concentration of approximately 9 $\log_{10}$ CFU ml^{-1}, as enumerated by plating on brain heart infusion agar (Difco).

Plant pathogens. *P. expansum* was obtained from W. Janisiewicz, (Agriculture and Food Research Center, U.S. Department of Agriculture, Kearneysville, W.V.) on potato dextrose agar (Difco). *G. cingulata* was obtained from W. Conway (U.S. Department of Agriculture, Beltsville, Md.) also on potato dextrose agar. These cultures were stored at 4°C, and subcultured every 14 days, by streaking on a fresh potato dextrose agar plate. Cultures for each experiment were prepared by growth on potato dextrose agar at room temperature (RT) under constant light, to maximize sporulation, for 10 days. Spores from the cultures were harvested for each experiment by pipetting 1 ml Tween 80 (0.2% vol/vol) (Aldrich Chemical Co., Milwaukee, Wis.) on to the surface of the culture. The lid was replaced on the petri dish and the dish gently shaken for 1 min. Using a pasteur pipette, the spore suspension was removed from the surface of the plate and added to a sterile test tube containing 0.5 g sterile glass beads (2 mm diameter). The tube was vortexed for 15 s to break up any clumps of spores in the suspension. A 1:30 dilution of this suspension (approximately 4 $\log_{10}$ spores ml^{-1}) was used for the inoculation of apples. A further 1:10 dilution of this suspension was made, and the spores in this solution were counted using a hemocytometer (AO Scientific Instruments, Buffalo, N.Y.).

Apple preparation. Unwaxed Golden Delicious apples (210 per experiment), obtained from a single Washington State grower, were removed from storage at 4°C and oriented on their sides on bench paper. Each apple was punctured using a sterile nail (3.7 mm by 80 mm) inserted to a depth of 10 mm. Puncturing fruit with a nail in this uniform manner produced an injury similar to that which might be sustained by a stem puncture and gave a standardized receptacle for inoculation. The apples were divided into three groups of 70. *P. expansum* or *G. cingulata* spore suspensions (25 μl aliquots) (approximately 4 $\log_{10}$) and 25 μl of the *E. coli* O157:H7 culture (diluted to 5 $\log_{10}$ in PW, to give a concentration of approximately 3 $\log_{10}$ CFU g^{-1} in the wound site) were added to each wound site on each apple, i.e., 70 apples were inoculated with either *P. expansum* and *E. coli* O157:H7 or *G. cingulata* and *E. coli* O157:H7. The third set of apples (n = 70) was inoculated with *E. coli* O157:H7 only and used as a control. Following inoculation, the 70 apples in each set were divided into two groups of 35. Each group of 35 apples was stored in one of two plastic tubs (18 cm by 32 cm by 39 cm; Rubbermaid, Wooster, Ohio). The tubs were covered in aluminum foil and stored at RT (22 ± 1°C) or 4°C for a total of 14 days.

Three separate trials were set up to investigate the effect of the timing of inoculation with the respective pathogens. In trial A, apples were punctured and inoculated with the plant pathogens 4 days prior to inoculation with *E. coli* O157:H7. In trial B, apples were punctures and inoculated with the plant pathogens and *E. coli* O157:H7 on the same day. In trial C, apples were punctured and inoculated with *E. coli* O157:H7 2 days (RT storage) and 4 days (4°C storage) prior to inoculation with the plant pathogens. Three separate experiments were performed for each trial.

Enumeration of *E. coli* O157:H7. *E. coli* O157:H7 populations in the wound sites on the apples were enumerated on days 0, 1, 2, 4, 7, 10, and 14. Three apples were tested at each sampling interval. The diameter of any rot visible around the wound site was recorded and the area around the site (approximately 5 g) removed using a sterile cork borer (27 mm diameter) and diluted 1:5 with PW. Samples were blended for 1 min at high speed in a stomacher (Seward 400 circulator; Seward Ltd., London, England) and filtered through a filter stomacher bag (Seward). The resultant filtrates were decimally diluted in PW as necessary, and 50-μl aliquots were spiral plated (Spiral Biotech, Beltsville, Md.) or 0.1-ml or 1-ml aliquots manually plated, when increased sensitivity was required, onto brain heart infusion agar. The plates were incubated at 37°C for 2 h, to permit the recovery of injured cells *(9)*, then overlaid with sorbitol MacConkey agar (Difco), and incubated for a further 18 h. Colonies were manually counted, and the $\log_{10}$ mean results recorded. Three representative *E. coli* O157:H7 colonies from tryptic soy agar–sorbitol MacConkey agar plates from each trial at each storage temperature were identified using a commercial latex test (RIM *E. coli* O157:H7 Latex test, catalog no. 24-250; Remel, Lenexa, Kans.) that identifies the O157 and the H7 antigens.

pH measurement. Broth pH was measured before and after the growth of *E. coli* O157:H7, on duplicate broth samples that were not used for inoculation purposes, using a Beckman Φ 40 pH meter (Fullerton, Calif.). Duplicate apples were removed for pH measurement at each sampling interval. The apple was cut in half at the site of the inoculation wound and the pH electrode (Corning, N.Y.; attached to a Corning 130 pH meter) placed directly on the wounded tissue.

Analysis of results. Means and standard deviations were determined using commercial spreadsheet software (Excel 97, Microsoft).

RESULTS

***E. coli* O157:H7 and plant pathogen populations.** The mean population of the *E. coli* O157:H7 overnight culture was 9.17 $\log_{10}$ CFU ml^{-1}, with a mean initial inoculum level of *E. coli* O157:H7 of 3.14 $\log_{10}$ CFU ml^{-1} in the apple wound sites. The suspensions of *P. expansum* and *G. cingulata* spores used for inoculations had mean populations of 3.89 and 4.18 $\log_{10}$ spores ml^{-1}, respectively.

Survival of *E. coli* O157:H7 in the presence of plant pathogens. Figures 1 to 3 depict the changes in *E. coli* O157:H7 populations in the presence of *P. expansum* and *G. cingulata* and also in the presence of no competing plant pathogen (control) in wounds on apples held at both storage temperatures during trials A, B, and C. Overall, *E. coli* O157:H7 numbers declined steadily in apples stored at 4°C. However, the organism was generally still detectable at low levels (1.00 ± 0.78 $\log_{10}$ CFU g^{-1}) at the end of the 14-day storage period. *E. coli* O157:H7 cells in apples stored at RT had different survival patterns depending on the timing of inoculation with the respective pathogens and the plant pathogen present. *E. coli* O157:H7 numbers increased by almost 1 $\log_{10}$ by the end of the RT storage period in the presence of *G. cingulata,* when the plant pathogen had

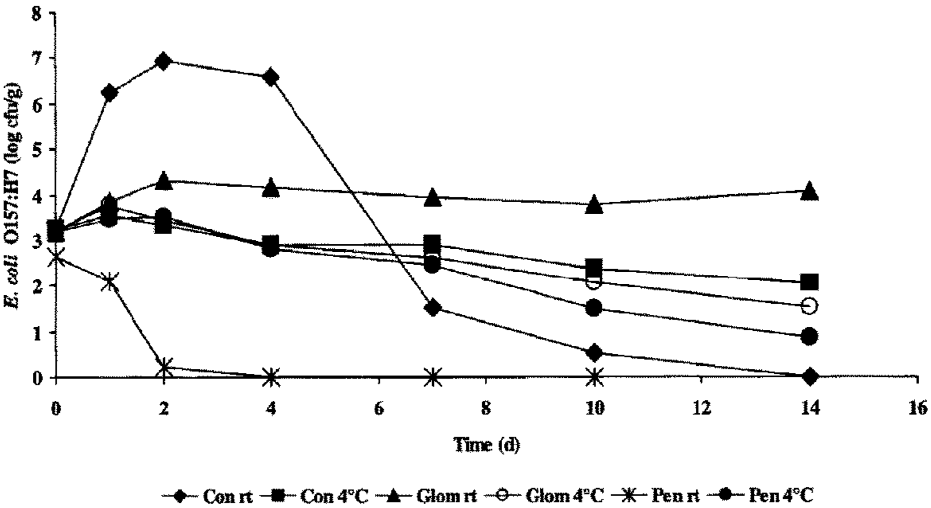

FIGURE 1. *Trial A: Survival of* E. coli *O157:H7 in the presence of* P. expansum *(Pen) and* G. cingulata *(Glom) in puncture wounds on apples stored at RT and at 4°C. Apples were injured and inoculated with Pen or Glom 4 days prior to inoculation with* E. coli *O157:H7.*

a 4-day start (Fig. 1). However, when both organisms were added at the same time (Fig. 2) or when the apples were inoculated with *E. coli* O157:H7 2 days before inoculation with *G. cingulata* (Fig. 3), *E. coli* O157:H7 numbers increased over 3 $\log_{10}$ to maximum populations of 6.31 ± 0.78 and 6.81 ± 0.18 $\log_{10}$ CFU g^{-1}, respectively. *E. coli* O157:H7 numbers generally declined to approximately 1 $\log_{10}$ CFU $g^{-1}$by the end of the 14-day storage period in the presence of *P. expansum.* Growth was observed for the first 1 to 4 days of RT storage when *E. coli* O157:H7 was added to the apples on the same day (Fig. 2) or 2 days prior to (Fig. 3) addition of this plant pathogen. It is reasonable therefore, to suggest that proliferation of the rot produced by *P. expansum,* as measured by increasing rot diameter over the 14-day storage period at RT (data not shown) was the cause of the elimination of the *E. coli* O157:H7 present.

One set of apples that had been inoculated with *E. coli* O157:H7 only were stored in the same manner as those inoculated with the plant pathogens for each trial, in order to determine the behavior of *E. coli* O157:H7 with no competing plant pathogens. In trials A and B opportunistic decay organisms, commonly *Penicillium* spp., grew at the wound sites and led to the destruction of the *E. coli* O157:H7 present (Figs. 1 and 2). Therefore no control could be reliably performed in these studies. However, in trial C, the control apples did not become infected with any opportunistic plant pathogen. In this trial *E. coli* O157:H7 populations achieved a plateau approximately 1 $\log_{10}$ lower than that observed for *E. coli* O157:H7 in the presence of *G. cingulata,* after 4 days storage under the same conditions (Fig. 3).

Changes in pH. *E. coli* O157:H7 grown in TSB with glucose reduced the pH of the broth from 7.28 ± 0.20 to 4.69 ± 0.15. The pH measurements of the wound sites on the apples at each sampling interval are shown in Figures 4 to 6. In trial A (Fig. 4) apples inoculated with *G. cingulata* attained a pH of 6.11 in the wound site due to proliferation of the plant pathogen prior to inoculation with *E. coli* O157:H7. Apples stored at 4°C generally showed little

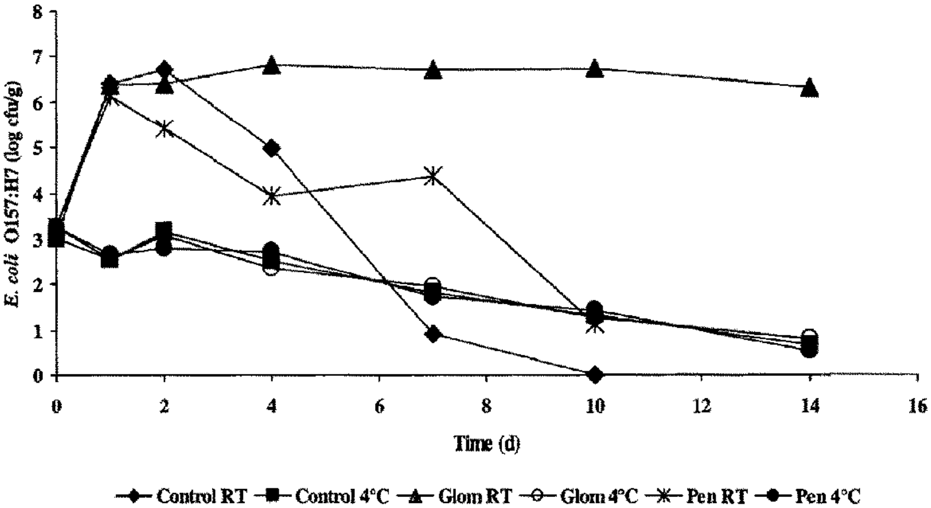

FIGURE 2. *Trial B: Survival of* E. coli *O157:H7 in the presence of* P. expansum *(Pen) and* G. cingulata *(Glom) in puncture wounds on apples stored at RT and at 4°C. Apples were injured and inoculated with* E. coli *O157:H7 and with Pen or Glom on the same day.*

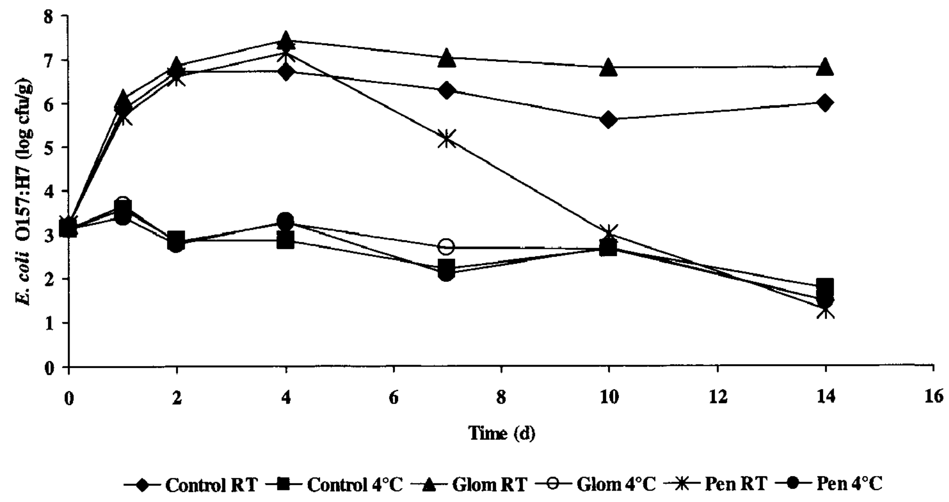

FIGURE 3. *Trial C: Survival of* E. coli *O157:H7 in the presence of* P. expansum *(Pen) and* G. cingulata *(Glom) in puncture wounds on apples stored at RT and at 4°C. Apples were injured and inoculated with* E. coli *O157:H7 2 days (for RT storage trials) and 4 days for (4°C storage trials) prior to inoculation with Pen or Glom.*

change in pH over the storage period, and no relationship was observed between changes in pH and concomitant changes in *E. coli* O157:H7 numbers at this storage temperature. Differences in pH were observed, however, in apples stored at RT and were associated with attendant changes in *E. coli* O157:H7 numbers. Control apples that showed evidence of rot due to opportunistic plant pathogens, and apples that had been inoculated with *P. expansum,* displayed a decline in pH from pH 4.0 to approximately pH 3.5 over the storage period. Apples that had been inoculated with *G. cingulata,* and to a lesser extent, control apples that were not infected with opportunistic fungi (trial C) demonstrated an increase in pH (from pH 4.10 ± 0.1 to pH 6.80 ± 0.2 and to pH 4.54 ± 0.1, respectively) over the same storage period. This indicates an association between the end point pH of the apples stored at RT and the final *E. coli* O157:H7 population present.

DISCUSSION

The results of this study demonstrate that *E. coli* O157:H7 can survive and grow in areas of injury on an apple and can extensively proliferate in the presence of *G. cingulata* when the apples are stored at RT. These findings indicate that the presence of *G. cingulata* rot on fruit is a cause for concern. *E. coli* O157:H7 populations increased 1,000-fold to attain a plateau of $>6 \log_{10}$ CFU g^{-1} in the area affected by *G. cingulata* rot. The very low infective dose of *E. coli* O157:H7 (between 5 and 50 CFU) *(20)* means that the presence of even one such piece of fruit in a batch destined for the production of unpasteurized juice would result in the contamination of approximately 1,000 liters of apple cider at a bacterial concentration that could cause foodborne illness. A batch of this size, which is relatively small, would be typical of that generated by small-scale producers who do not pasteurize their cider.

E. coli O157:H7 was shown to survive and grow in the presence of *G. cingulata,* regardless of the timing of inoculation with the respective organisms. The scenario of a dropped piece of fruit sustaining injury that permits the entry of human pathogens such as *E. coli* O157:H7 (e.g., from animal feces, contaminated water, or dust from nearby pastures), in addition to plant pathogens such as *G. cingu-*

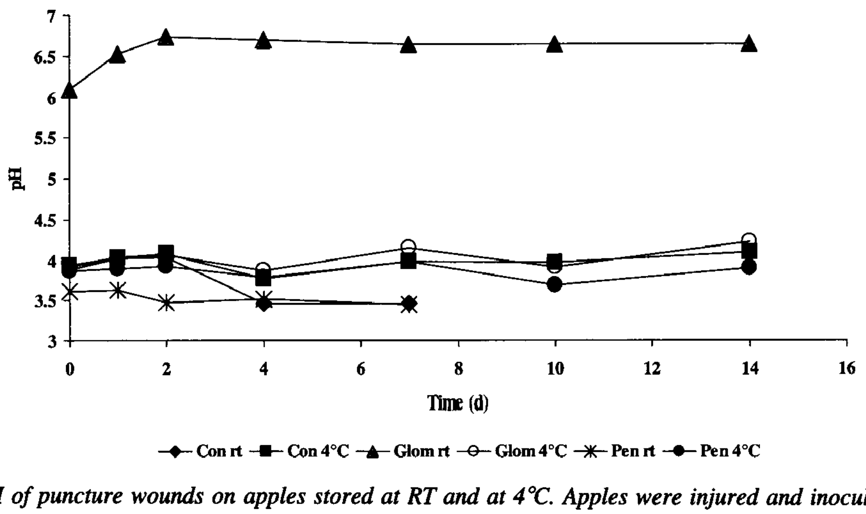

FIGURE 4. *Trial A: pH of puncture wounds on apples stored at RT and at 4°C. Apples were injured and inoculated with* P. expansum *(Pen) or* G. cingulata *(Glom) 4 days prior to inoculation with* E. coli *O157:H7.*

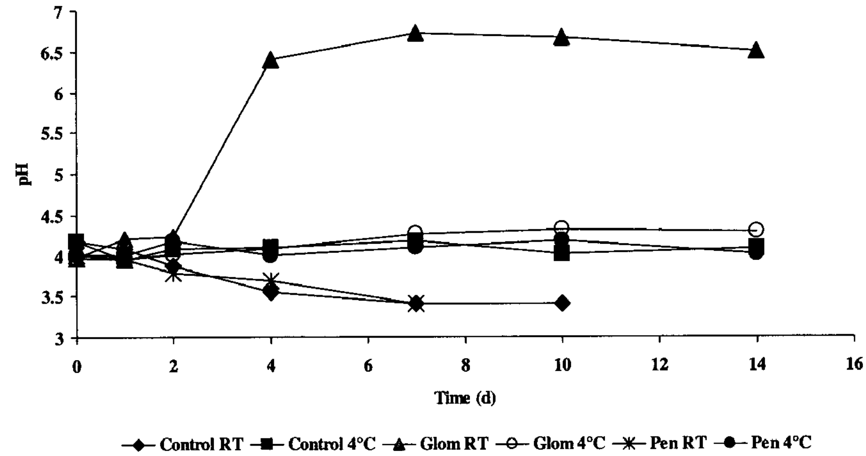

FIGURE 5. *Trial B: pH of puncture wounds on apples stored at RT and at 4°C. Apples were injured and inoculated with* E. coli *O157: H7 and with* P. expansum *(Pen) or* G. cingulata *(Glom) on the same day.*

lata, is not an unlikely one. Also, *G. cingulata* can infect apples by direct penetration of the cuticle, producing a lesion through which human pathogens such as *E. coli* O157: H7 can later enter *(19).* The ability of *E. coli* O157:H7 to survive and grow in areas of injury on an apple, also reported by Janisiewicz et al. *(13),* underlines the necessity for the exclusion of dropped fruit and the culling of rotted fruit from that destined for the production of unpasteurized apple cider.

The ability of *E. coli* O157:H7 to proliferate in the presence of *G. cingulata* is correlated with an attendant rise in pH of the affected site on the apple. The reason for this rise in pH is unclear. It is possible that the enzymatic activity of *G. cingulata* led to the production of alkaline end products, or perhaps the organism could metabolize the malic acid present, resulting in lowered acidity and increased pH. The reasons for the increase in pH observed were not investigated in this study. This effect is worthy of further study, as is the possibility that other plant pathogens be screened for pH changes during growth on apples, to determine if other plant pathogens exist that have similar effects on the physiology of fruit.

E. coli O157:H7 was shown to increase in population 1,000-fold over the 14-day storage period at RT, though no rot development was observed. Similar findings were reported by Janisiewicz et al. *(13),* who reported a maximum population of 6 to 7 $\log_{10}$ CFU per wound, regardless of the initial inoculum size. Small puncture wounds, such as those that have been used in this study and that by Janisiewicz et al. *(13),* could be easily overlooked during the sorting process—yet such apples would have the potential to contaminate a large volume of unpasteurized cider. Other studies performed in our group have shown that bacteria present in areas of injury and around the stem and calyx of the apple can form biofilms that cannot be removed by conventional washing steps or indeed any of several experimental washes tried *(18).* Such findings suggest that the only way to assure the safety of apple cider is to include an intervention step in the production process that can assure a 5-$\log_{10}$ reduction of the target pathogen, i.e., *E. coli* O157:H7, in the finished product.

Cold storage has been shown to reduce the survival of *E. coli* O157:H7 in areas of injury on apples in the current study. No rot development was observed at 4°C in any trial

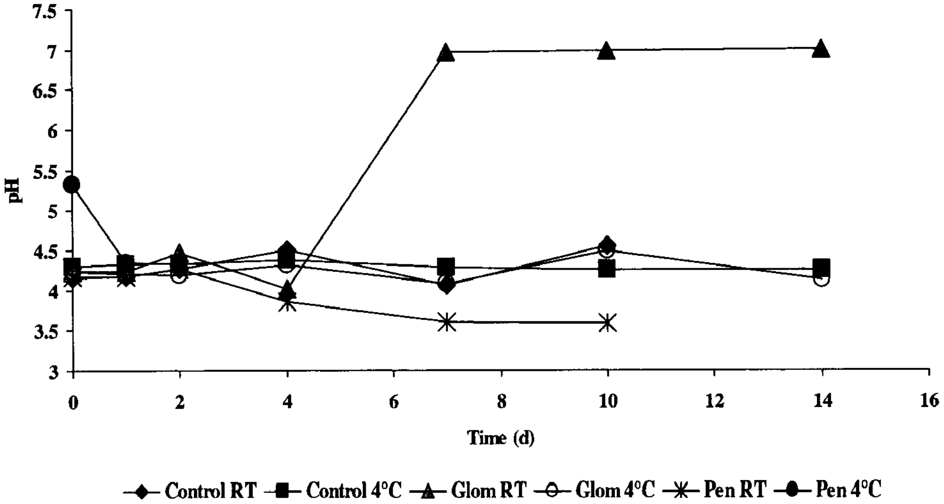

FIGURE 6. *Trial C: pH of puncture wounds on apples stored at RT and at 4°C. Apples were injured and inoculated with* E. coli *O157: H7 2 days (for RT storage trials) and 4 days for (4°C storage trials) prior to inoculation with* P. expansum *(Pen) or* G. cingulata *(Glom).*

performed. The minimum temperature for the growth of most molds that cause rotting during the storage of apples is below 0°C, so their development will generally not be prevented by the use of refrigeration *(10)*. It may be that the storage period in the current study was too short for the proliferation of the plant pathogens to commence. However, it is unlikely that *E. coli* O157:H7 could survive extended storage in apples at refrigerated temperatures, therefore the industry practice of storing apples at 2 to 3°C *(17)* is a prudent one.

The findings of this study underline the importance of careful culling of apples and omission of drops as critical control points in the production of unpasteurized apple cider. *G. cingulata* also has the ability to cause disease in the orchard or during storage in fruits as diverse as apples, pears, peaches, quinces, and cherries *(15)*, and processors of such commodities should be aware of the implications of this plant pathogen that is worldwide in distribution *(1)*. To our knowledge, the proliferation of *E. coli* O157:H7 in association with an opportunistic plant pathogen such as *G. cingulata* has not been demonstrated before. The information presented here should be useful to orchard managers and fruit juice producers who will be alerted to the potential consequences of this plant pathogen.

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